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Phylogenetic Position of the Dicyemid Mesozoa Inferred from 18S rDNA Sequences

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Abstract. The dicyemid mesozoa, obligate symbionts in the cephalopod kidney, are simply organized multicellular animals. They have long been the subject of phylogenetic debates. Some authors have suggested that dicyemids represent an offshoot from an early metazoan ancestor. Other workers considered them to be degenerated progeny of higher metazoa, possibly parasitic trematodes. We determined the almost complete nucleotide sequences of 18S rDNA in two species of dicyemid, *Dicyema orientale* and *Dicyema acuticephalum*, isolated purely from cephalopod urine. We compared these sequences with sequences determined in the present study from three flatworm species, as well as with a variety of eukaryote sequences obtained from databases. The phylogenetic trees reconstructed with the use of the neighbor-joining, maximum-parsimony, and maximum-likelihood methods indicated that the dicyemids belong among the triploblastic animals (Bilateria). However, we cannot firmly establish the position of the dicyemids within the Bilateria because we cannot ignore the problem of long branch attraction between the myxozoans, dicyemids, nematodes, and acoel flatworms. The present results favor the hypothesis that the dicyemids do not represent an early divergent metazoan group, but rather a group degenerated from a triploblastic ancestor.

Introduction

The dicyemids are simply organized multicellular animals consisting of an outer layer of 20–40 ciliated somatic cells and an inner core of one long axial cell. They are obligate symbionts in the kidney of cephalopods. Their life cycle is complex; the asexually produced vermiform embryos increase the population in the host, while the infusoriform embryos arising from fertilized eggs pass out of the host body with the urine and are thought to infect another cephalopod host (Brusca and Brusca, 1990).

The dicyemid mesozoans have long been the subject of a phylogenetic controversy (Brusca and Brusca, 1990; Willmer, 1990). They were at first considered to be an extant link between the Protozoa and the Metazoa (Hyman, 1959). However, the resemblance of their complex life cycles to those of parasitic trematodes has led some authors to propose that the dicyemids are descended from an established metazoan group and that their simple body organization results from degeneration attributable to parasitism (Nouvel, 1948; McConnaughey, 1951; Stunkard, 1954; Ginetsinskaya, 1988). Others still view the simple body construction of dicyemids as truly primitive and hold that the group represents an offshoot from early divergent metazoa (Dodson, 1956; Hyman, 1959; Lapan and Morowitz, 1974).

The phylogenetic relationships of eukaryotes have recently come under intense scrutiny in the light of new molecular data. Phylogenetic analyses using nucleotide sequences of 5S rRNA suggested that a dicyemid (*Dicyema misakiense*) diverged early among such lower metazoa as sponges, cnidarians, and flatworms (Ohama *et al.*, 1984; Hori and Osawa, 1987). But phylogenetic trees based upon comparisons of about 120 sites in the nucleotide sequences of 5S rRNA were different from those inferred from longer nucleotide sequences of 18S or 28S ribosomal RNA (Field *et al.*, 1988; Christen *et al.*, 1991; Katayama *et al.*, 1993; Wainright *et al.*, 1993; Kobayashi *et al.*, 1993). We have sequenced 18S ribosomal RNA

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genes (18S rDNA) in two species of the dicyemid mesozoa. Our comparison of the nucleotide sequences of small-subunit rDNA for a variety of organisms indicates that the dicyemids belong among the triploblastic animals.

Materials and Methods

Biological materials

We determined almost the entire sequence of 18S rDNA in two species of dicyemid mesozoans and three species of turbellarians (Platyhelminthes). Pure samples of the dicyemids *Dicyema acuticephalum* and *Dicyema orientale* were collected from the urine of *Octopus vulgaris* and *Sepioteuthis lessoniana*, respectively (Furuya *et al.*, 1992a). Specimens of *Convoluta naikaiensis* (Acoela) and *Planocera multitentaculata* (Polycladida) were collected on the shore near the Ushimado Marine Laboratory. Specimens of *Dugesia japonica* (Tricladida) were obtained from the brook near the Ushimado Marine Laboratory. All were frozen quickly and kept at −80°C until use.

In addition to the sequences of the above five species, we used the sequences of 23 eukaryotes—including animals, protists, plants, and fungi—for which almost complete 18S rDNA sequences were available in databases. The species used and their accession numbers are as follows: *Paramecium tetraurelia* (Ciliophora), X03772; *Oxytricha nova* (Ciliophora), X03948; *Crypthecodinium cohnii* (Dinozoa), M64245; *Theileria annulata* (Apicomplexa), M64243; *Sarcocystis muris* (Apicomplexa), M64244; *Hartmanella vermiformis* (Rhizopoda), M95168; *Saccharomyces cerevisiae* (Fungi), J01353; *Filobasidiella neoformans* (Fungi), X60183; *Arabidopsis thaliana* (Plantae), X16077; *Volvox carteri* (Plantae), X53904; *Beroe cucumis* (Ctenophora), D15068; *Trichoplax adhaerens* (Placozoa), L10828; *Anemonia sulcata* (Cnidaria), X53498; *Scypha ciliata* (Porifera), L10827; *Henneguya* sp. (Myxozoa), U13826; *Caenorhabditis elegans* (Nematoda), X03680; *Moliniformis moliniformis* (Acanthocephala), Z19562; *Schistosoma mansoni* (Trematoda), X53047; *Crassostrea gigas* (Bivalvia), X60315; *Artemia salina* (Crustacea), X01723; *Sagitta crassa* (Chaetognatha), D14363; *Asterias amurensis* (Asteroidea), D14358; and *Xenopus laevis* (Vertebrata), X04025.

DNA isolation

Genomic DNA was extracted by the method described previously (Wada *et al.*, 1992). In brief, the frozen samples were lysed in TE buffer (10 m*M* Tris-HCl, 0.1 *M* EDTA, pH 8.0) containing 0.5% sodium dodecyl sulfate. After digestion with proteinase K (100 μg/ml) at 50°C for 3 h, DNA was extracted with phenol and precipitated in ethanol and an equal volume of 5.0 *M* ammonium acetate. Samples resuspended in TE buffer were further purified by RNase A digestion (20 μg/ml) at 37°C for 1 h followed by ethanol precipitation.

Amplification of 18S rDNA

The 18S rDNA was amplified by the polymerase chain reaction (PCR; Saiki *et al.*, 1988) in an Air Thermo-cycler 1645 (Idaho Technology). Almost the entire length of 18S rDNA was amplified using synthetic oligonucleotides, 5′-CTGGTTGATCCTGCCAG-3′ (primer 0) and 5′-CCTTGTTACGACTT-3′ (primer 10) as the terminal primers. Amplifications were performed in 50 μl of 50 m*M* Tris-HCl (pH 8.5), 250 μg/ml BSA, 2 m*M* Mg^{2+}, with 0.2 m*M* each dNTP, 50 p*M* primers, template DNA (5–10 ng), and 2 U Taq DNA polymerase (TOYOBO). The temperature regimen for 35 cycles was 20 s at 94°C, 30 s at 50°C, and 90 s at 74°C.

Determination of DNA sequences

After purification of the amplified DNA by electrophoresis in a 0.8% agarose gel, the nucleotide sequence was directly determined by dideoxy chain-termination (Sanger *et al.*, 1977) using Sequenase ver 2.0 (USB) and [^{35}S]-dATP (Amersham). All DNA samples were sequenced in both directions and from several separate amplifications with terminal primers (0 and 10) and internal primers. The internal primers used were primer-1 (5′-CCGGAGAGGGAGCCTGA-3′), primer-2 (antisense of primer-1), primer-3 (5′-CAGCAGCCGCGGTAATT-3′), primer-4 (antisense of primer-3), primer-5 (5′-GCGAA-AGCATTTGCCAA-3′), primer-6 (antisense of primer 5), primer-7 (5′-GAAACT(TC)AAAGGAAT-3′), primer-8 (antisense of primer-7), and primer-9 (5′-ACGGGC-GGTGTGT(AG)C-3′). The positions corresponding to these primers in 18S rDNA sequences are shown in Figure 1. The continuity of the DNA fragments was confirmed by overlapping of the sequences.

Phylogenetic analyses

Sequences were aligned manually on the basis of maximum nucleotide similarity (Fig. 1). Alignment gaps were inserted to account for putative length differences between sequences. Some regions could be confidently aligned and were presumed to be homologous. However, we could not unequivocally determine the optimal alignment for the regions containing deletions, insertions, or highly variable sequences. We excluded positions from the analysis according to the following rule: Positions where a gap was present for any taxon were not used in analyses. In the present study, we found this simple rule alone adequate for excluding the regions of ambiguous homology (the regions where two or more equally optimal alignments were present) from the analysis because in those

regions alignment gaps were always serially inserted in many sequences. The phylogenetic trees were reconstructed using the PHYLIP package version 3.5c (Felsenstein, 1989) and fastDNAML (Olsen *et al.*, 1993). Tree-building procedures used were the neighbor-joining (Saitou and Nei, 1987), the maximum-parsimony (Fitch, 1971), and the maximum-likelihood (Felsenstein, 1981). For the neighbor-joining analysis, evolutionary distance values were calculated by the formula of Jukes and Cantor (1969). The degree of support for internal branches of the trees in the neighbor-joining and the maximum-parsimony trees was assessed by bootstrap levels of support (Felsenstein, 1985) determined by 500 bootstrap repetitions.

Results

In *D. acuticephalum, D. orientale, Convoluta naikaiensis, Dugesia japonica,* and *Planocera multitentaculata,* almost the entire length of 18S rDNA was amplified by PCR from the genomic DNA. The sequence (1500–1700 bp) was determined directly from PCR products. The sequences have been deposited in databases (GSDB, DDBJ, EMBL, and NCBI) under the following accession numbers: D26529 for *D. acuticephalum;* D26530, *D. orientale;* D17558, *Convoluta naikaiensis;* D17560, *Dugesia japonica;* D17562, *Planocera multitentaculata.* To infer the phylogenetic position of the dicyemids within the eukaryotes, we aligned the almost complete nucleotide sequences of 18S rDNA of the above five species with the 23 eukaryote sequences we obtained from databases. Metazoan taxa were chosen to represent phyla broadly; protozoan taxa were chosen to represent the more recently derived groups. Figure 1 shows a sample of the alignment for 9 out of 28 species included in the present analysis. This alignment reveals that throughout the eukaryotes the sequences are highly conserved in some regions and highly variable in others. After exclusion of the regions of ambiguous homology, 1070 sites (Fig. 1) remained for phylogenetic inference. Phylogenetic trees shown were reconstructed by the neighbor-joining (Fig. 2), the maximum-parsimony (Fig. 3), and the maximum-likelihood (Fig. 4) analyses.

Among the phylogenetic trees reconstructed by the three methods, the topologies were largely congruent with one another, though branching with low bootstrap support within the metazoan lineage showed somewhat conflicting arrangements. The metazoans—including triploblasts (Bilateria), diploblasts, dicyemids, and a myxozoan—formed a monophyletic assemblage in the three trees. Within the metazoan assemblage, triploblasts formed a discrete monophyletic unit together with the mesozoa and the Myxozoa. The branches of triploblasts were in general longer than those of other taxa.

The grouping of the dicyemids with the triploblastic animals was supported by a bootstrap value of 100% in both the neighbor-joining (Fig. 2) and the maximum-parsimony (Fig. 3) analyses (because of the enormous computation time required, bootstrapping was not performed in the maximum-likelihood analysis). In the trees reconstructed by the three methods, dicyemids were grouped with *Caenorhabditis elegans* (a nematode), *Henneguya* sp. (a myxozoan), and *Convoluta naikaiensis* (an acoel flatworm), though bootstrap confidence level for this grouping was low.

To corroborate the inclusion of the dicyemids in the triploblastic lineage, we analyzed subsets of taxa shown in the present paper as well as several different sets of taxa including some of the following species (the accession numbers for 18S rDNA data are shown in parentheses): *Cryptomonas phi* (X57162), *Babesia bovis* (M87566), *Tetilla japonica* (D15067), *Sycon calcaravis* (D15066), *Mnemiopsis leidyi* (L10826), *Tripedalia cystophora* (L10829), *Paraspadella gotoi* (D14362), *Antedon serrata* (D14357), *Strongylocentrotus intermedius* (D14365), *Balanoglossus carnosus* (D14359), *Oikopleura* sp. (D14360), *Branchiostoma floridae* (M19571), *Homo sapiens* (X03205). In all sets of taxa analyzed, triploblasts formed a monophyletic unit and the dicyemids were placed within the triploblastic clade with high bootstrap confidence level. Grouping of *Caenorhabditis, Convoluta, Henneguya,* and *Dicyema* were consistently observed. *Trichoplax,* another enigmatic animal whose phylogenetic position is controversial (Brusca and Brusca, 1990; Willmer, 1990), was always positioned outside the triploblastic assemblage, confirming analyses by Wainwright *et al.* (1993).

Discussion

The present molecular phylogenetic study based upon comparisons of nucleotide sequences of 18S rDNA shows that triploblastic animals form a monophyletic assemblage within the metazoan subtree and that the dicyemid mesozoa are an ingroup of the monophyletic unit of triploblastic animals. Monophyly of triploblastic animals has repeatedly been shown in previous molecular phylogenetic analyses with 18S or 28S rDNA sequences (Field *et al.*, 1988; Christen *et al.*, 1991; Wainright *et al.*, 1993; Kobayashi *et al.*, 1993; Smother *et al.*, 1994). The present analysis confirms the recent claim by Smother *et al.* (1994) based upon 18S rDNA sequences that the Myxozoa are closely related to the triploblastic animals.

As in phylogenetic trees previously constructed on the basis of rDNA sequences (Christen *et al.*, 1991; Wainright *et al.*, 1993; Smother *et al.*, 1994), *Trichoplax,* which had once tentatively been grouped in the phylum Mesozoa (see Brusca and Brusca, 1990), was positioned within the

```
                                                    50                                                 100
D.acuticephalum     ATGCATGCGTAAGCTCATGCTCT-CTAATGAGCGAAACCGCAGACGGCTCATTAAATCGGACATAACTTACTTGATAATCTC-ACACGACGGTGGATAAC
D.orientale         nnnnnn.TA....T........-..........G.....-.............................................................
C.naikaiensis       .......T.....TAT..CT----TG..AA..T.......GA T.............A.TT.CT.TC..AAG.C.G.GTGA-CATTCTACAA.A...G.
D.japonica          nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn..G-.T.........T.A.A.C...G.T...AGA....TAA..T.TTT.C.ACAA......
P.multitentaculata  nnnnnnnnn.C..TA..CATCA--TATTACGAT.......GA.T.............A.CT..GGT.CCT.G...GC.G..---.ATTACA........
A.sulcata           .......TC....TAT.A..A.--T.GTACT.T.....T..GA.T.............A.TT..CGT...T.....TG.ACG-TTTACTACT........
P.tetraurelia       .......TC....TAT.AATAG---..TAC..T.....T..GA.T...........A.A.TT...GT...T......G.GCA---.ATTA.A........
A.thaliana          .......T.....TATGAA.GAATTC.GACT.T.....T..GA.T.............A.TT...GT..GT.....GG.AA.---TACTACTC.......
S.cerevisiae        .......TC....TAT.A..AA.-T..TAC..T.....T..GA.T.............A.TT..CGT...T......G.TC.TTT..T..ATG.T.....

                                                   150                                                 200
D.acuticephalum     TGTTGTAAATCTAGAGCTAATACATGCGTACAGCTTCTCG-----------AAGCGCAATTATTAGA---------------------------------
D.orientale         ...........................AG.A........-----------.................---------------------------------
C.naikaiensis       ..CGC...C-.GC.T.T....G.T...AG.TT....GGGA--------GACTGAGA.TT...CG...T----ATCCACCGGGGAGCTCTGAAACACCGAG
D.japonica          ...G....T............TT.TAAAGA.TGCCG.GACT--AACGAAGCG.T.G.T........T-CAAAACTAAC-CTGTCGCAAGAACCA-----
P.multitentaculata  ...G.G..T..................ACCA.AGCCA.GACCGCAAGGGAATG.....T........T-CAAAACCAAC-CGGCTTC------------
A.sulcata           C..G....T...................A.G..TCC.GACTTCT--GGAAGGGAT.T.T........TTCAAAACCAATGCGGGTTCT-----------
P.tetraurelia       C..G....T...................C.A.TACCGGACGC---AAGAATGGTA...T........TTTAA--CCAT---------------------
A.thaliana          C..A....T..............G...AACA.A.CC.GACTTAT--GGAAGGGA....T........TAAAAGGTCGACGCGGGCTCTG----------
S.cerevisiae        C..G....T..................T..A.ATC..GACCCTTT-GGAAG.GAT.T.T........TAAAAAATCAATG-T--CTTCG-----------

                                                   250                                                 300
D.acuticephalum     ---------------ATTAGTTGACTCTGGATAAACTTGCTGATCACA-------GnAGTGCCTGCGACGTATTAATT-AGATGTCCGCCCCATCAATTT
D.orientale         ---------------...................T..........G------G.CT....-..T.......C....-...................G
C.naikaiensis       ACCCAGGCGATGATG.A.C.CGT.G..GT.TG.....GAAA....GT.CAAAGGCCGT.AAGGCTG.TGACG.AGTC.GC...TG.T.AG.T.....CC.
D.japonica          ---------------C..T.A.............CT..A.GT...GT.C--GACCATTA..T.GA....AA..CTC..G.AG..G.T.A..T........
P.multitentaculata  -----GGC--CTGTTTG.T.A...................AGC.GAT.GCATGG-CCAT.A...G.........CTT.C-.A.....T....T........
A.sulcata           -----GCC--CGGTGC..T.G...T..ATAG...CTGAT.GA...G..T---GGCCTT.C..TG....T..T.C.T.C-.A..T..T....T.....C..
P.tetraurelia       --------------C.CAT.G...A...TAG...CT.A.....C.G.GT----------.ATAC.T.GTAA..C.T.C-.AG.T..T....T....GC..
A.thaliana          ------GC--T--TGC.CT.A...T..AT.....CTCGA.G....G..T--GGCCTCT....TG......C..C.T.C-.A..T..T....T.....C..
S.cerevisiae        ----------GA-CTC..T.A...T..ATA....CT..T.GA...G..T---GGCCTT....TG....T.GT.C.T.C-.A..T..T....T.....C..

                                                   350                                                 400
D.acuticephalum     TCGACGGTGAGGTAGTGGCTTACCGTGGTTA-CAACGGGTAACGG-GAATCAGGGTTCGATTCCGGAGAGGGAGCATGAGAAATGGCTGCCAGATCTATG
D.orientale         .T.............................-.............-......................................................
C.naikaiensis       AT......AGT...-A.CAC..........G-TG....C......G.......................A.....C.......C....A.T.C...C.A.
D.japonica          ....T...A..A.CAAA.......A.....G-T............G.......T.....................C.......C....A...C...ACG.
P.multitentaculata  A..TT...AG.T..CCT..C....A....G.T.............G.....................................C....A.....A...C.
A.sulcata           ...AT...A....GT.........A.....-.........G....A....T........................C.......C....A...C...C.A.
P.tetraurelia       ....T...AGT...T...AC....A...CAG-TC...........A....T........................C.......C....A.........A.
A.thaliana          ....T...AG.A.......C....A....GG-T......G.....A....T........................C.......C.........C...C...
S.cerevisiae        ....T...AG.A.......C....A.....T-.............G....A........................C.......C....A...C...C.A.
                                                                                 Primer 1,(2)

                                                   450                                                 500
D.acuticephalum     GATTGCAGCAGGTGCGT-AAATTATCCACTTTTGGCAT----AAAGAGATAGTGATCATAAATAACGGATCCGGGCAT-----TTGCCAGGAGATCGTAA
D.orientale         .................A-...................----..............................T.....-----...TTCA..........
C.naikaiensis       ..A.........CA..C-......C...A...-..A.C----GGG...G......CG.G......TCAT.TAA.C.T.A----A..GTTTATA..GAG..
D.japonica          ..AG........C...AT......C...AC.A-...CGTAGCCCT...G......CA..........AATATG....C.---AG.G.TTT.ATA..T.C..
P.multitentaculata  .T..........C...C-......C.....C--....C----GGG...G......CG.A........AATA.G..T.T.TTTATAG...CC.TA..T.G..
A.sulcata           ..AG........C...C-......C...A.CC..A.TC----.GG...G......CA.G..C.....AATA.A....T.-TTGTAA.T.TT.TA..T.G..
P.tetraurelia       ..AG........C....-......C...A.CCC.ATTC----GGG...G......CA.G.....G.AAC..GT.CGGG-TTTCC-.TT.C.G...T.C..
A.thaliana          ..AG........C...C-......C...A.CC..A..C----GGG...G......CA..........AATA......TC-TTTCGA.T.T..TA..T.G..
S.cerevisiae        ..AG........C...C-......C...A.CC.AATTC----.GG...G......CA...........ATA.A....CC-ATTCGG.T.TT.TA..T.G..
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Figure 1. Nucleotide base sequences of 18S rDNA from two dicyemids, *Dicyema acuticephalum* and *Dicyema orientale*, and three flatworms, *Convoluta naikaiensis, Dugesia japonica*, and *Planocera multitentaculata* The sequences are aligned to that of small-subunit rDNA of a diploblast, *Anemonia sulcata;* a protist, *Paramecium tetraurelia;* a plant, *Arabidopsis thaliana*, and a fungus, *Sacchromyces cerevisiae*. A period indicates that the base at that position is identical to that in *D. acuticephalum*, a hyphen indicates a gap, and an "n" indicates an undetermined site. The 1070 positions used for phylogenetic inference are shown by single lines above the alignment. The positions corresponding to the internal primers are shown by double lines below the alignment. The primers whose numbers are in parentheses are antisense to the sequence shown here.

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                                                                      550                                               600
D.acuticephalum      TGAAATAACT-GTAAAAGCTTTAATGAATG-CAATTGGAGGGCAAGTCTGGTGCCAG-CAGCCGCG-CAATTCCAGT-TCCAATAGTGTATACTAAAGTT
D.orientale          .......TC-................C.-.........................-.......-.........-.....................
C.naikaiensis        ......GGA.-CG.......CC.....TGAC.G.G.A..................-..C...---T..A....CC..T.CA..C....CGATG....
D.japonica           ...G.ACTA.TT....TA.....TCA.G.AT........................-........-T.......C-........C.....T.......
P.multitentaculata   ...GT.C..C-T....CC.A....C..G.AC........................G........-T.......C-........C.....T.......
A.sulcata            ...GT.C.AC-T....TC......C..GGAT.C......................-........GT.......C-........C.....T.......
P.tetraurelia        ...G....G.-CC...TC.G.A..C..T.AA........................-........GT.......C-........C.......T.....
A.thaliana           ...GT.C.A.-C....TC.C....C..GGAT.C......................-........GT.......C-........C.....T.T.....
S.cerevisiae         ...GT.C.A.-.....TA.C....C..GGAA........................-........GT.......C-........C.....T.......
                                                                                  Primer 3,(4)

                                                                      650                                               700
D.acuticephalum      GCTGCAGTTGAAAAGCTCGTAGTTGGATCTCGGTGTGCTAGTACGTAATCGCG-TGCTAGTAGGCCTTTG--CTATAGTTAG--ACTATG----------
D.orientale          ..................................CT......CG..C..A-..--..AA............--CTGTAGT.G..T---------------
C.naikaiensis        .....GT..A.....................G--.T..G..T.TGA..CATC..C.AT--AC.CATTGCGCGTA..G..GCCTCG..GCCCATCCTT--CG
D.japonica           .T.CG....A................A.AT.GA.GAAATG.T.TATAT.A--A.T.TA.GACT.ATTA.C.AGA.CCT--.CCTTCTCCGTCGTGATATTC
P.multitentaculata   .T.......A.....................GA.GA.TAG..CCA-C.GGTTG.CC..AT.GCTA-.AC.TGTGGC.CAGCCTGC.TAG.CGGTGAT--TG
A.sulcata            .T.......A...................CT....G...G--CACG.CCGGTC..CC..A...GT.T.AC..G---CCG.GCC.CTCT.C.TCGCAAAGAC-
P.tetraurelia        .T.......A................A.AT...AGTCAG.TACTA..TGG.T.TTC.TC...TA.TTAA.TGAT.C.CCG.CTAC.A.CCCTTTTGCGCT-
A.thaliana           .T.......A................A.C..T..GA..G--..CG.CCGGTC..CCTT.G..GT.-.A...G---TCG.C.T.TCC..TC.GTCGGCGATA
S.cerevisiae         .T.......A................A.CT.T..GCCCG--..TG.CCGGTC..A.TT.TTCGT.T-AC..GAT.TCCAACG.GGC..T.CCTTCTGGCTA

                                                                      750                                               800
D.acuticephalum      ---------------------------------------------------GC--TTTACCTTGAACAAA-ATAGAGTGCTTAAGGC-AAGCATTCTGCTTG-A
D.orientale          ----------------------------------------GCTACG..--...............-................-.....-A........A.
C.naikaiensis        CATCGATGATATTAGTTGCACTTTGTTGTGACGACTAGTGGAAACGGTGTT..C..T....GT...-T.......T.C..A..-..C.T..GG.A...C.
D.japonica           GTTAAGTGCACTTTATTGGGATCTTTTACAA--TAACCGACAA-------G.....T.....A...-T............A..G.G..T.ATGCT.GCAT
P.multitentaculata   TTTTGGTGCTCTTAATTGAGTGCCTTAATTGCCCG---GCCA-------CG.....T.....A...AT.G.......C..AT.-.G..CCAA...C..A.
A.sulcata            CGCGTGTGCTCTTGACTGAGTGTGCGCGGGAGTTGCGA-----------CG.....T.....A...-T.......T.C..A..-.G..-CAGC.....A.
P.tetraurelia        ---TTAGGGTTGCAGCTGGGCGAGTAG------------ACAA--------...........A...-T.......T.CC....-.G.T-C.TC..CG.A.
A.thaliana           CGCTCCTGGTCTTAATTGGCCGGGT-CGTGCCTCCGGCG----------CTG....T.....G...-T.........C..A..-.....C.A.GCTC..G.
S.cerevisiae         ACCTTGAGTCCTTG--TGGCTCT-TG-GCGAACCAGGA-----------CT.....T.....A...-T.......T.C..A..-.G..G.AT....C.A.

                                                                      850                                               900
D.acuticephalum      TATCTAAGCATGGAATAATAGAATAAGAC-TTTTCTA--------TTGGTT-ACG-A-TAGTAAAAGTAATG-TTAACAGAGACAGCCGGGGGCATCCGT
D.orientale          .....C.................A.....-......G--------.....T..C-.G..............A.............A.........T...
C.naikaiensis        ..ATAA.............G..GA.T...-C.GAG.CAACGTTTTGAACGCGGT.GCTGTTGCTCG..G...A..G.A..GA....G.........TT..
D.japonica           AT..GTT............GA........-..CGG.TTTATTTTG......TT..-.AAC--TG........A....A.........-.........AT..
P.multitentaculata   C.GA.CTCG..CC......CA..A--....-..CnG.TCTATTTTG..-...TT..G.AC.--TG........A....G..G.....A.........T...
A.sulcata            ..CA...............G.....G...-..GGG.TCTATTTTG......TCT.G.ACC--TG........A....G..G.....TT........T...
P.tetraurelia        ..CA.T.............G.....G...-..GGG.C---TTTTG......TTA.G.C.----TG.......A....T..G.....AT........TA..
A.thaliana           ..CA.T.......G....C.TC...G..T-..CGA.CCTATTGTG....C.TCG.G.TC----GG.......A.......G.....T.........T...
S.cerevisiae         ...A.T...................G...G...GG.TCTATTTTG......TCTAGGACC---.TC......A....T..G...G.T..........G..

                                                                      950                                               1000
D.acuticephalum      ATTGCTCCGTTATAGGTGAAATTCGTAGATCGGTGCAGGACGTA-CTACAGCGAA-GCATTTGCCAAC-ATGTTTTCATTAATCAAGAACGACAGTTGGA
D.orientale          .....A...C..G...........................A.-..........-...........G-..............................
C.naikaiensis        ......A.....G...........T.G.....TG...A...AA.-....T.....A...........GA............................A..
D.japonica           ..GCTGGT.C..G...........TG...C.ATCAGCA....A.A....T.....A...........GA...........................CA..
P.multitentaculata   .....GGT.GG.G...........T.G....ATC...A..GCC--..........A...........GA...........................CA..
A.sulcata            ...-.GTT..C.G...........T.G...TTAC.A.A....A.-....T.....A...........GA............................A..
P.tetraurelia        ...TAATT..C.G...........T.G...TTA.TA.A...TA.-..TAT.....A...........GG............................A.G
A.thaliana           ...T.ATA..C.G...........T.G...TTA..A.A....A.-.A..T.....A...........GG..............................G
S.cerevisiae         ...CAATT..C-G...........T.G...TTA.TG.A...TA.-....T.....A...........GG.C..........................A.G
                                                                           Primer 5,(6)
                                                                      1050                                              1100
D.acuticephalum      GTATCGAAGTCGATTAGATCTCGTCGTAGTTCCAACTATAAACGATGCCAACTAGCACTCCGCTGATAATATTTGGATACATTAGCGGGTGGCTTCCGGG
D.orientale          ..................................................................A....G..--TTTG............T........
C.naikaiensis        ....TA...AG........AC.C.....A...T...C......TT.A......TA.GA.TT.TCCC.GTCCC-.CTCG..TGGGCAA.AATTTAA.....
D.japonica           .G.......A....C....AC....C......TG..C......T......T..GA.GAGTTAGC..AGGATA..TA..CATC.T..TA.AA.TCA.....
P.multitentaculata   .GT......A....C....AC....C......TG..C.................G...A....T..CG.T.GCGA.TTCGATCC.A....CA..C......
A.sulcata            .GC......A....C....AC....C.....AT...C............G.....GGA..A.AGAG.GT...-.....GACC.CTTT..CAC...AG...
P.tetraurelia        .G...A...A....C....AC.........CTT..........T..A..G.....AG...G.AA.GGT.ATA-.ATTAGTCCCTTTC..CAT.G.AA.A.
A.thaliana           .GC......A....C....AC....C....CT....C............G..C..GGA..A..G...GT.GC..AT.GGAC.CC..T..CAC...AT.A.
S.cerevisiae         .G.......AT...C....AC.........CTT..C......T.....G....--GA..G.G..G.GT.T...TA..GACCC.CTC...AC...A..A.
```

```
                                                                  1150                                           1200
D.acuticephalum    AAACCAAAGTCTTTGGGTTCCGGGGGAAGT-ATGGTTGCAAAGCTGAAACTTAAAGGAATTGAC-GGAAGGGCACCACCAGGAGTGGAGCTTGCG-CTCA
D.orientale        ..............................-.......-...................-.....-.............-......-....C....-..-.
C.naikaiensis      ..........T.A.T...............-...........ATC...................-.........................C....-..T.
D.japonica         ............A.................G.................................-....................-...-C...--..T.
P.multitentaculata ..............................-.................................T....A....-...A...........C....G..T.
A.sulcata          ..........T........T......G...-.................................-.........................C....-..T.
P.tetraurelia      ...T................T.....G...-.....C....G......................-.........................C....G..T.
A.thaliana         ...T......T...............G...-.....C....G......................-.........................C....G..T.
S.cerevisiae       ...T................T.....G...-.....C....G......................-.........................C....G..-.
                                                                   Primer 7(8)

                                                                  1250                                           1300
D.acuticephalum    ATTTGACTCAACGCAGAAAAACTCACCCGGGCCGAACACAGTGAGGATTGACAGACTGATAGCTTTTTCTTGATACTGTGGGTAGTGGTGCATGGCCGTT
D.orientale        ............A.......................................................................................
C.naikaiensis      ............A.G.GG.........A..T..AG...TCT.A............TA.TATA.-C.C..A.A..CAGA....CG................
D.japonica         ............A.G.G...TT..C.....T...G....T...............T........C.........T.G......G................
P.multitentaculata ............A.G.G.............C...G....T...............T...A....C.........T.G......G................
A.sulcata          ............A.G.GG.........A..T..AG...T..GA............T...G....C.........T..A.....G................
P.tetraurelia      ............A.G.GG.....T...A..T.AA....TG.ATG...........T...A....C.........T..A.....G................
A.thaliana         ............A.G.GG.....T...A..T..AG...T...A...................G....C.........T..A.....G................
S.cerevisiae       ............A.G.GG.........A..T..AG.....A.A............T...G....C.........TT......G................

                                                                 1350                                            1400
D.acuticephalum    -GTTGGTTGGTTGGAGTGATTTGTCTGGTTTATTCCGATAACGAACGAGACTCAGACTTACTTACTAGTCGTACGATTCTAGTTGT---------ACGAC
D.orientale        -.........-.........C.........A.......................AG.C...........T..TT....G.T.G.CG-------AT-.A..
C.naikaiensis      -C..A...A.-.........C.........A...................TCTT.G.CA.T.A.A...GAAAG.AG.A.CGAGGTA---TTGCAA.-AG.
D.japonica         -C..A....-.....C.........A....A.....................TA....G..A.A....GTATTT...T..AC.CGTCAAATAAT-.AC.
P.multitentaculata -C..A....-.....C.............A.....................TAG.C....A.A....ACACTC..C.ATT.GTG--TGAGTGC-....
A.sulcata          -C..A....-...................A......T............CTTA..C.G..A.A....TACG.CCA..GC.A..G----GCAAC-TA..
P.tetraurelia      -C..A....-...................A...................CTTA..C.G..A.....TTGCTTG.GAACAACAG------GTA-TA..
A.thaliana         -C..A....-.....C.............A......T............CTCAG.C.G..A.....CTACGT.GAGGC.TCCC.--TCACGGC-..G.
S.cerevisiae       TC.CA....-................C..A...G...............CTTA..C....A.A....G..G.T.GCA.TTGCTG----GTTAT-.C..

                                                                  1450                                           1500
D.acuticephalum    TACTAAGAAGGATCAGTG---------TGAAAACACTTGAAAATGAGCAATAACAGGTCTGTGATTGCCCTTAGA-CGTTCGGGGC-GCACGCGTGCTAC
D.orientale        ...-T...G-........---------.TT...ACAC.T..............................-.....-..........T.A.....C.....
C.naikaiensis      .T..T...G.C.CTGT.CCGAATTTAAAT.CGGA.AGC...GTGAG.................-.........-T....T..A.T......C.....
D.japonica         .T..T.....A..A.A.A------GCG.CT.CGT.AA......A...................-.....A....-T..C.....GC.......C.....
P.multitentaculata .T..T..GG...CA....-----GCAT.C..GC..TAC..............................-..........-T..C......C.......C.....
A.sulcata          .T..T...G...CTGT..------GTG.TT..C..AAGTC.GGAAG.................-..........-T....T....C.......C.....
P.tetraurelia      .T..T...G...CT.TGT-----ATGTAAGTGCATGGAAGTTTAAG..................-.....C....-...C.T....C.......C.....
A.thaliana         .T..T...G...CT.TG.----CCGTT.AGGCCA.GGAAGTTTGAG..................-..........-T....T....C.......C.....
S.cerevisiae       .T..T...G...CT.TC.----GTTTCAAGCCGATGGAAGTTTGAG..................-..........A.....T....C.......C.....

                                                                 1550                                            1600
D.acuticephalum    AATGAAGAAAGCAGAGGG-------GGTTTTGCTTGGAAAAGCGAACTAAGCCT-TAAAATTTCTTCGTGGCAGGAATCGAGGCTTGTAATTATTCCTCG
D.orientale        ....G........T..-------T..C.......C.....A.G..G....-...............A.............................
C.naikaiensis      .C..T.CTCTA.....T.-TGAAAAACC.AAT..A.AG.GGATTGGGA..T.ACAG..CGGAGAAATT.T.AG..G....T.CA......C.T..G.A..
D.japonica         ....GCAGTTC..AC.A.--TAATACC..AAC..A.A..T..TTGGGC..T.T.G.G...CACTG....AA....G..T.T.AA...........T.A.A
P.multitentaculata .C....T.GC.T.AC.A.--TTATTCTCC.GATCC.A..GG.TCGGGC..C.TGT.G...CC.A......A....G....G................C..
A.sulcata          .C...C..TGT..AC.A.----TCTCTCC...GCC.A..GGT.TGGG...T.T.C.C...CA..G.....CTG..G..A..TCA...C....T..GA..T
P.tetraurelia      .C...CACGTT...C.A.CTTATTTACC.G.C.CGAA.GGGCACGGGA..T.T.G.T.GGACGTG.....CTG..G..A..TCT...C......AGA..T
A.thaliana         .C...T.T.TT..AC.A.----TTCACACC.TGCC.AC.GGC.CGGG...T.T.-.G.......A.....ATG..G..A..TCA...C....G..GG..T
S.cerevisiae       .C...C.G.GC...C.A.----TCTAACC...GCC.AG.GGT.TTGG...T.T.G.G...C.C.G.....CTG..G..A...CA...........G...T

                                                                  1650                      1684
D.acuticephalum    TGCACAAGGAATTCCTAGTAATCGC-AGGTCATTAGCCTGCAATGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTAC
D.orientale        .........................-...........................................................
C.naikaiensis      ..A..CC.........C....AT.G-.A....AC.A.T.CT.T.........................-............
D.japonica         ..A..G...............G...A.A...nnnnn.nnT..T.nnnnnnnnnnnnn..........................
P.multitentaculata ..A..G...............G...-.A.....A.A.T...GT........................................
A.sulcata          ..A..G...............G...-GA.....C...TC..GT........................................
P.tetraurelia      ..A..G..........T....G.A.-.......C......TGC...A...................................C.
A.thaliana         .CA..G...............G...-GA.....C...TC..GT...C....................................C.
S.cerevisiae       .CA..G...............G...-.A.....C...T...GT.......................................G
                                                                                 Primer(9)
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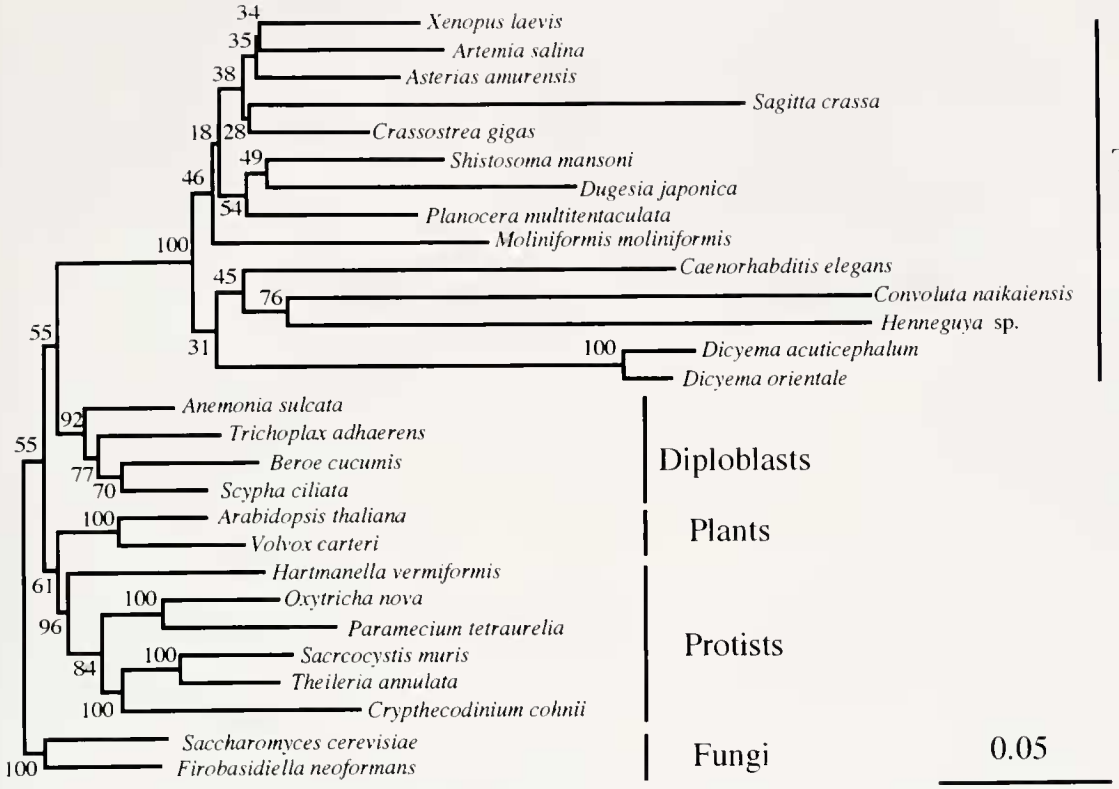

Figure 2. Neighbor-joining trees showing the phylogenetic position of the dicyemids among 28 representative eukaryotic taxa. The tree was reconstructed on the basis of the pairwise distances of Jukes and Cantor (1969) using DNADIST and NEIGHBOR programs (PHYLIP package, version 3.5c). The tree was rooted by using *Saccharomyces cerevisiae* as an outgroup. Branch lengths are proportional to the scale given in substitutions per sequence position. The percentage of 500 neighbor-joining bootstrap replicates is shown at the node the value is supporting.

diploblastic assemblage. This study does not support a close relationship between *Trichoplax* and mesozoans.

These sequence data do not firmly establish the position of the dicyemids within the triploblastic assemblage. In the phylogenetic trees obtained by three different methods, the dicyemids formed a monophyletic unit with the myxozoans, nematodes, and acoel flatworms. They are all considered to be early divergent groups in one widely accepted phylogeny. The early divergence of acoel flatworms in triploblastic evolution has been suggested by Katayama *et al.* (1993) from comparisons of partial 18S rDNA sequences. However, the myxozoans, dicyemids, nematodes, and acoel flatworms were all represented by a long branch in the phylogenetic trees (a high nucleotide substitution rate). Hence we cannot ignore the possibility that these long branches produce artifactual groupings within the triploblastic assemblage (Van de Peer *et al.*, 1993).

With regard to the topology of triploblast phyla, the present phylogenetic trees contradict those of some previous analyses of 18S rDNA sequences in some points. The Platyhelminthes did not form a monophyletic unit as previously shown by Katayama *et al.* (1993). Monophyly of the deuterostomes and the protostomes has repeatedly been shown in the molecular phylogeny of 18S rDNA (Wada and Satoh, 1994; Raff *et al.*, 1994; Halanych *et al.*, 1995), but neither group was monophyletic in all trees of the present analysis. Triploblastic phyla are poorly resolved in the molecular phylogenetic trees of 18S rDNA; *i.e.*, nodes defining phyla are not supported by high bootstrap values. Therefore, the topology of the trees depends largely on the choice of taxa. Philippe *et al.* (1994) have showed that by eliminating rapidly evolving species from the analysis, discrepancies between molecular and traditional phylogeny partly disappear and bootstrap values rise at some nodes. Since we only intended to show the placement of the dicyemids firmly within the triploblasts, we chose taxa representing a broad spectrum of eukaryote phyla without regard to consistency with the traditional view of triploblast phylogeny.

There have been opposing views on the role of the dicyemid mesozoa in the story of metazoan evolution. Some

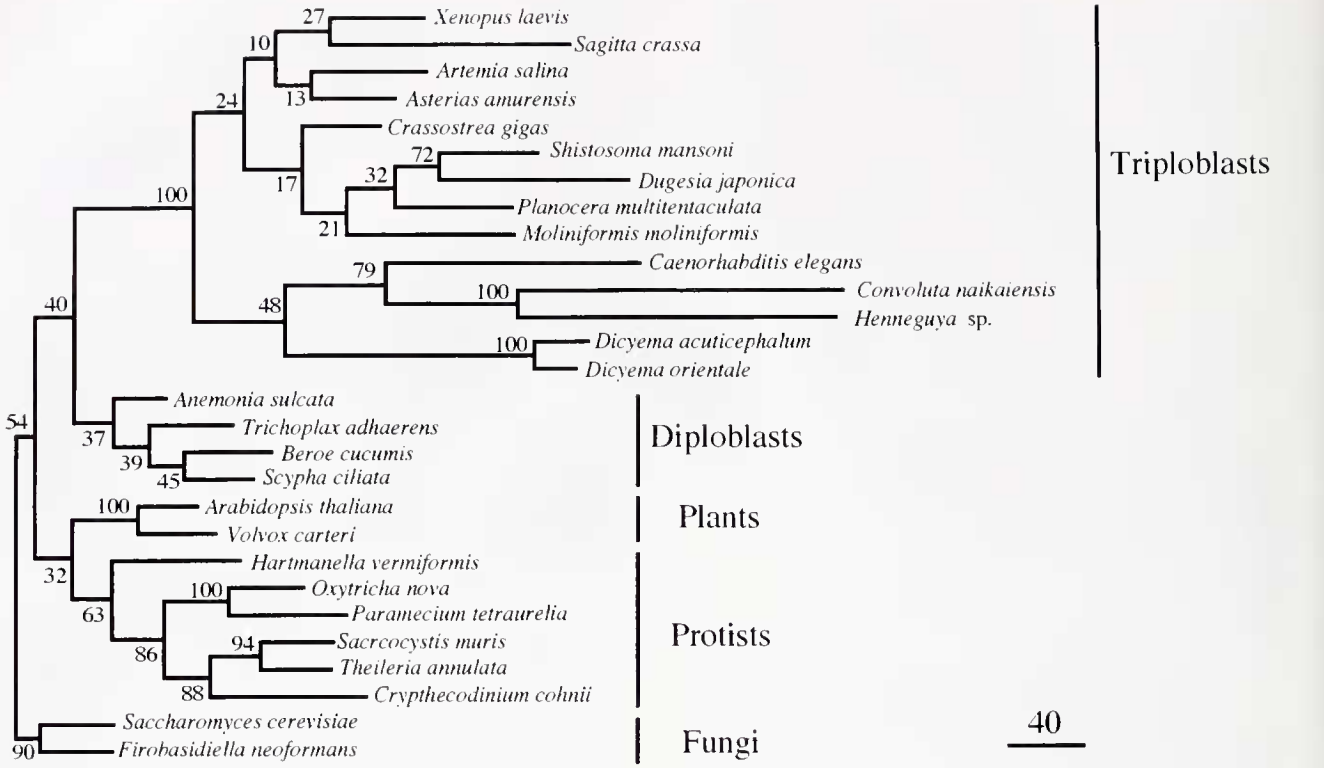

Figure 3. The consensus tree obtained using the maximum-parsimony algorithm with bootstrap resampling (DNAPARS, SEQBOOT, and CONSENSE programs of PHYLIP package, version 3.5c), showing the phylogenetic position of the dicyemids among 28 representative eukaryotic taxa. The percentage of 500 parsimony replicates is shown at the node the value is supporting. The tree was rooted by using *Saccharomyces cerevisiae* as an outgroup. Branch lengths are proportional to the scale given in number of substitutions (a total of 2807). This tree is different from the three most parsimonious trees in the positions of *Crassostrea gigas* and *Moliniformis moliniformis* within the assemblage of the coelomate triploblasts.

authors have proposed that the dicyemids are a missing link between unicellular organisms and multicellular animals (Dodson, 1956; Hyman, 1959; Lapan and Morowitz, 1974; Ohama *et al.*, 1984), while others have claimed that they are an animal group degenerated as a result of parasitism (Nouvel, 1948; McConnaughey, 1951; Stunkard, 1954; Ginetsinskaya, 1988). The phylogenetic trees inferred from comparisons of nucleotide sequences of 5S rRNA suggested that the dicyemids emerged first among the metazoa examined and that triclad flatworms, nematodes, cnidarians, and sponges followed, in that order (Ohama *et al.*, 1984, Hori and Osawa, 1987). This suggestion does not, however, accord with the present result and the previous inferences about metazoan phylogeny based upon 18S and 28S rDNA sequences (Field *et al.*, 1988; Christen *et al.*, 1991; Wainright *et al.*, 1993; Kobayashi *et al.*, 1993). Discrepancies are partly ascribable to differences in the methods used to infer phylogenetic relationships. In contrast to the 18S and 28S rDNA trees reconstructed by the neighbor-joining, maximum-parsimony, and maximum-likelihood methods, the above 5S rRNA trees have been reconstructed by unweighted and weighted pair group methods using arithmetic averages (UPGMA and WPGMA, respectively), which are valid under the assumption that rates of nucleotide substitution are constant among taxa analyzed (Sokal and Mitchener, 1958). However, the essential point is that the 5S rRNA is too small to contain signal sufficient to allow precise inference of phylogenetic relationships. Because of large standard errors, sequential orders of branching of the dicyemids, flatworms, nematodes, cnidarians, and sponges shown in the above 5S rRNA trees appear to be statistically insignificant. Recently Halanych (1991) analyzed the sequence data of 5S rRNA with the maximum-parsimony method. The phylogenetic tree obtained was inconsistent with phylogenies based on 18S and 28S rDNA data, and few nodes in the tree were supported by bootstrap value at a significant level.

The present results do not appear to support the proposition that the dicyemids are a truly primitive group linking unicellular organisms with multicellular metazoa. Instead, our results favor the view that the dicyemids are

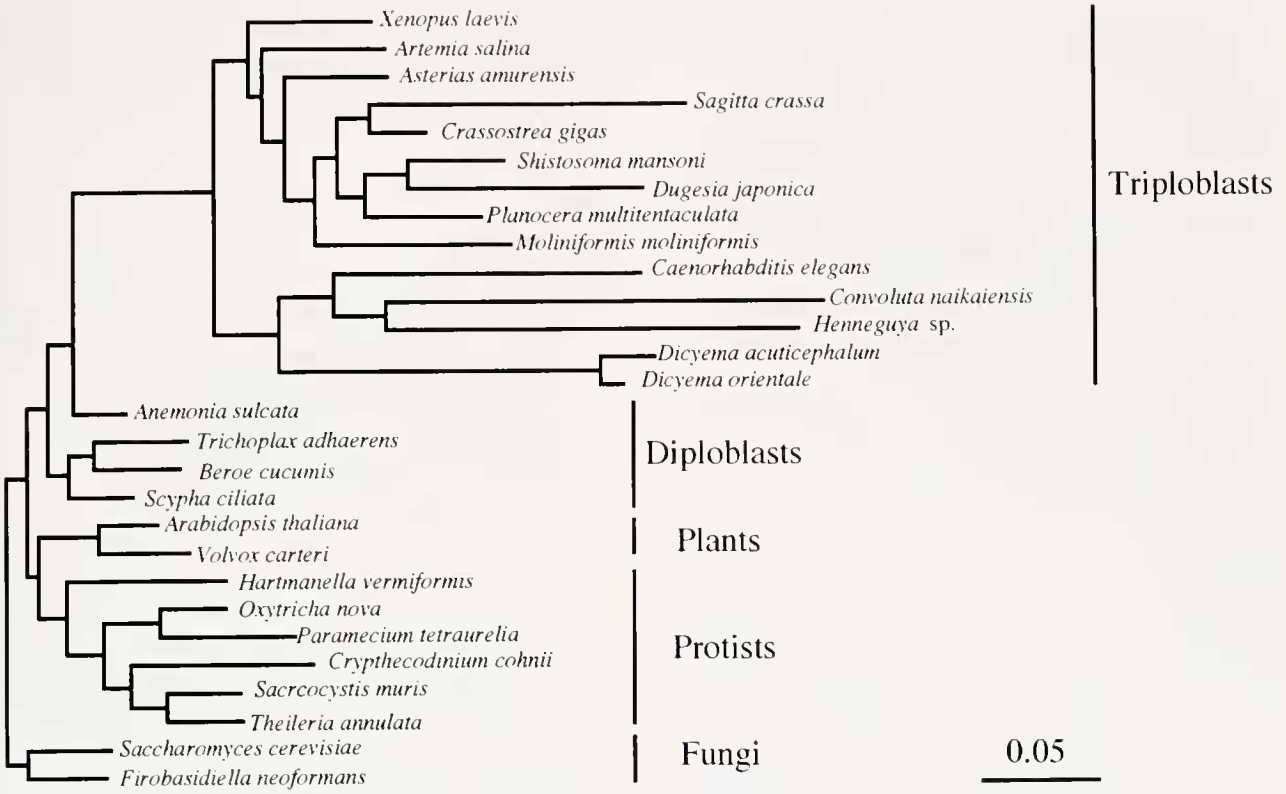

Figure 4. Maximum-likelihood tree showing the phylogenetic position of the dicyemids among 28 representative eukaryotic groups. The tree was obtained using the fastDNAML algorithm with a transition/transversion ratio of 1.48, which gave the best maximum-likelihood score (ln likelihood = −11909.45548). Branch lengths are proportional to the scale given in substitutions per sequence position. Because of the long computation time (more than 12 h per replication), bootstrapping was not performed.

degeneratively simple animals descended from a more complex triploblastic ancestor. Recent close observations of dicyemid development (Furuya *et al.*, 1992b, 1994) do not contradict the present inference; spiral cleavage, a determinative mode of cell division, and the formation of stereoblastula-like structure through epiboly, as seen in the development of vermiform and infusoriform embryos, are reminiscent of flatworms. Myxozoans are also regarded as an extreme example of the degeneration characteristic of parasitic evolution (Smother *et al.*, 1994). At present we can say little about the ancestor from which the dicyemids were derived. Molecular analyses that include other lower turbellarian groups (for example, the Catenulida and the Nemertodermatida) and the Orthonectida, a group tentatively included in the Mesozoa, will provide further information for understanding the phylogenetic position of the dicyemids.

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